



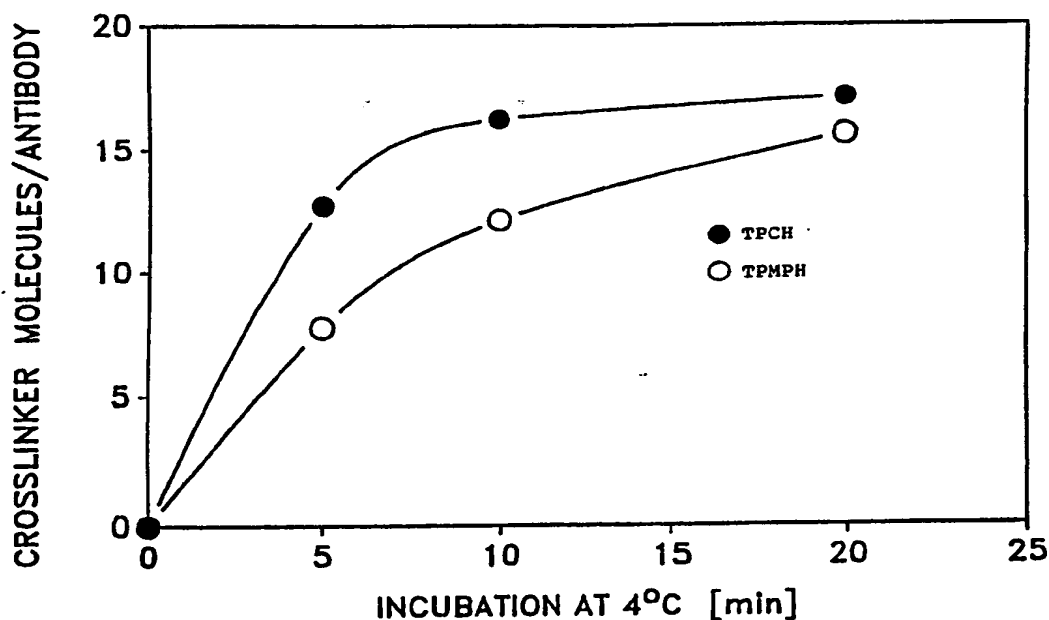
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(21) International Application Number: PCT/US90/01201 (22) International Filing Date: 13 March 1990 (13.03.90) (30) Priority data: 322,214 13 March 1989 (13.03.89) US (71) Applicant: GEORGETOWN UNIVERSITY [US/US]; 37th & O Streets, Northwest, Washington, DC 20057 (US). (72) Inventors: ZARA, Jane, J. ; 1342 Irving Street, Northwest, Washington, DC 20010 (US). WOOD, Richard, D. ; 2216 40th Place, Apartment ,1, Washington, DC 20007 (US). BREDEHORST, Reinhard ; 2617 42nd Street, Northwest, Apartment ,202, Washington, DC 20007 (US). VOGEL, Carl-Wilhelm ; 4100 Massachusetts Avenue, Northwest, Apartment ,1403, Washington, DC 20016 (US).		(74) Agents: OBLON, Norman, F. et al.; Oblon, Spivak, McClelland, Maier & Neustadt, Fourth Floor, 1755 South Jefferson Davis Highway, Arlington, VA 22202 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>

(54) Title: SITE-SPECIFIC HETEROBIFUNCTIONAL CROSSLINKING REAGENTS



(57) Abstract

Site-specific heterobifunctional crosslinkers of the formula: X-COCH(NH₂)-Y-Z, where X is a carbonyl reactive group, Y is a variable length spacer, and Z is a thiol reactive group, are useful for the specific labelling of biomolecules or bioaffecting molecules.

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DescriptionSite-Specific Heterobifunctional Crosslinking ReagentsTechnical Field:

The present invention relates to site-specific
5 heterobifunctional crosslinking reagents, biomolecules or
bioaffecting molecules to which the present crosslinking
reagents are bound, and kits containing biomolecules or
bioaffecting molecules to which the present crosslinking
reagents are bound.

10 Background Art:

Heterobifunctional crosslinking reagents are widely used
for linking effector molecules to biomolecules such as
glycoproteins including antibodies, lectins, enzymes, and
response modifiers.

15 In particular, immunoconjugates result from the chemical
coupling of monoclonal antibodies with various effector
molecules, which may include toxins (e.g., ricin A chain;
reviewed by Moller, in, Immunol. Rev., p.62, Copenhagen
(1982)), biological response modifiers (e.g., cobra venom
20 factor; reviewed by Vogel in Immunoconjugates. Antibod Conlug
adioima and Therapy of Cancer, pp. 170-188 Oxford Univ. Press
1987)), and low molecular weight drugs (e.g., doxorubicin;
reviewed by Sela and Hurwitz in Immunoconjugates. Antibody
Conjugates in Radioimaging and Therapy of Cancer, pp. 189-216,
25 Oxford Univ. Press (1987)). The potential--of such
immunoconjugates for the development of novel anti-cancer
therapeutics and imaging techniques has been studied by many
investigators and has been reviewed by C.-W. Vogel in
Immunoconjugates, Oxford Press (1987),

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Several methods for coupling effector molecules to monoclonal antibodies are known. Currently used methods for the synthesis of immunoconjugates employ heterobifunctional crosslinking reagents which contain one amino-reactive residue (e.g., succinimidyl ester) and one sulfhydryl-reactive residue (e.g., pyridyl disulfide). An example of such heterobifunctional reagents is N-succinimidyl-3-(2-pyridylthio)propionate (SPDP) which is described in Biochem. J., vol. 173, pp. 723-737 (1978). Upon incubation of antibodies with SPDP, the crosslinking reagent is coupled via an amide bond to one of the primary amino groups of the antibody, thereby introducing a pyridyl disulfide moiety to which a sulfhydryl-containing effector molecule can be coupled.

One major problem of this technique, however, is the random distribution of amino groups throughout the entire antibody molecule including the antigen binding region. Thus, the crosslinking agents may bind to the antibody at a site close to the binding region of the antibody and, thus, interfere with the binding of the antibody. Therefore, use of the currently available heterobifunctional crosslinking agents diminishes the antigen binding capability of the resulting immunoconjugates and, thereby, limits their efficacy as therapeutic and diagnostic agents.

More recently, the reaction of amine-containing molecules with the carbohydrate region of antibodies has been investigated for the purpose of preparing antibody derivatives, see D.J. O'Shannessy, Int. Soc. Biorecognition Tech., vol. 3, pp. 4-6 (1988).

PCT Patent Application W087/06837 discloses linking amine-derivatives of folic acid to antibodies which contain an oxidized carbohydrate moiety.

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U.S. Patent 4,671,958 discloses the use of crosslinkers, one of which contains a hydrazine derivative, for the purpose of reacting with oxidized antibody carbohydrate moieties. However, only enzymatically cleavable crosslinkers are disclosed.

Japanese Patent Application J63-57569 discloses bridging agents which are hydrazide compounds of the formula $X-SS-A-CONHNH_2$, where X is 2-pyridyl or 4-pyridyl and A is a C_1 to C_6 divalent hydrocarbon group (e.g., $-CH_2CH_2-$, $-CH_2CH_2CH_2-$). These compounds are used to link effector molecules to the sugar chain of an antibody. For example, the antitumor agent methotrexate is linked to an antitumor antibody via the sugar chain to give an antibody/methotrexate complex to be used in target therapy. Linking enzymes with antibodies for enzyme immunoassays is also disclosed.

However, the crosslinkers of J63-57569 react relatively slowly with biomolecules so that they require the antibody to be exposed to the oxidizing medium for a prolonged period of time. The prolonged exposure of the antibody leads to undesirable side reactions which decrease the binding function of the antibody. Further, the crosslinkers of J63-57569 are not sufficiently water soluble to make their handling and manipulation easy.

In addition, the crosslinkers of J63-57569 are achiral and, thus, the possibility of optical isomers does not exist. Since many of the molecules which are to be attached to crosslinkers exist as optically pure isomers, crosslinkers which can exist as optical isomers might possess some advantages, such as site specificity.

Thus, there remains a need for site-specific heterobifunctional crosslinkers which couple to biomolecules or bioaffecting molecules with a high efficiency, react with

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biomolecules and bioaffecting molecules with a high rate, and possess a high water solubility.

Disclosure of the Invention

It is an object of the present invention to provide
5 site-specific heterobifunctional crosslinkers which couple with biomolecules and bioaffecting molecules with a high efficiency.

It is another object of the present invention to provide site-specific heterobifunctional crosslinkers which react with
10 biomolecules and bioaffecting molecules at a high rate.

It is another object of the present invention to provide site-specific heterobifunctional crosslinkers which possess a high water solubility.

It is another object of the present invention to provide
15 biomolecules and bioaffecting molecules to which the present site-specific heterobifunctional crosslinkers have been attached.

It is another object of the present invention to provide kits which contain biomolecules or bioaffecting molecules to
20 which the present site-specific heterobifunctional crosslinking agents have been attached.

These and other objects of the present invention which will become apparent during the course of the following detailed description have been achieved by crosslinking agents
25 having the formula:



wher in X is H_2NNH- , $H_2NNHCONHNH-$, $H_2NNHCONH-$, $H_2NO(CH_2)_nNH-$, $H_2NOCO(CH_2)_nNH-$, or $H_2N(CH_2)_nNH-$, where n is an integer of 2 to

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6; Y is a divalent group having 1 to 20 carbon atoms, which may be interrupted by heteroatoms, and may be substituted with hydroxyl, carboxy, sulfonate, phosphonate, and quaternary ammonium groups; and Z is dithiopyridyl, thiolacetate, or
 5 maleimide.

Brief Description of the Drawings

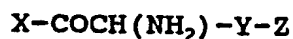
A more complete appreciation of the present invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to
 10 the following detailed description when considered in connection with the accompanying drawings, wherein:

FIGURE 1 shows a comparison for the incorporation of TPCH versus TPMPH into human monoclonal IgM antibody 1688; and

FIGURE 2 compares the kinetics of incorporation of
 15 TPCH and TPMPH into human monoclonal IgM antibody 1688.

Best Mode for Carrying Out the Invention

The present invention relates to site-specific heterobifunctional crosslinkers having the formula:



20 where X may be H_2NNH- , $H_2NNHCONHNH-$, $H_2NNHCONH-$, $H_2NO(CH_2)_nNH-$, $H_2NOCO(CH_2)_nNH-$, or $H_2N(CH_2)_nNH-$, where n is an integer of 2 to 6.

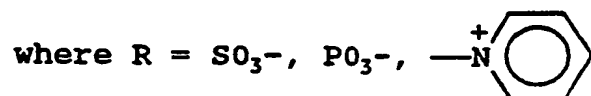
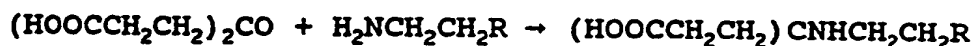
Y is a divalent group having 1 to 20 carbon atoms, which may be interrupted by heteroatoms, and which may be
 25 substituted by hydroxyl, carboxy, sulfonate, phosphonate, and/or quaternary ammonium groups. The purpose of the spacer region Y is to separate the reactive groups X and Z of the linker. This region may vary in length between one and twenty

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carb n atoms. In a preferred embodiment, Y is a C₁-C₃ alkylene moiety. When Y is interrupted by heteroatoms, it is preferably interrupted by 1 to 3 atoms selected from: O, N, and S.

5 The C₁-C₂₀ divalent organic group for Y may also be substituted by 1-5 functional groups. One reason for this substitution is to impart greater hydrophilicity to the overall molecule. Thus, as the carbon atom content increases, the number of substituents is desirably increased in order to
 10 adjust the aqueous solubility of the molecule. Suitable functional groups include hydroxyl, carboxylate, sulfonate, phosphonate, and quaternary ammonium salts. These functionalities can all be introduced by conventional methods, e.g., via reductive amination of keto residues. The quaternary
 15 ammonium salts are preferably of the formula -NR₁R₂R₃·X-, wherein each of R₁ to R₃ is independently selected from C₁-C₇ alkyl or hydrogen, and X is an anion such as chloride, bromide, etc.

In one embodiment, the ends of the Y region precursor may
 20 be carboxylic acid residues, to facilitate the coupling to the amino acid and Z regions of the linker. A number of diacids containing keto functionalities are available and may be used as convenient precursors to the Y region. For example, sulfonate, phosphonate, or pyridinium residues can be
 25 introduced into 4-ketopimelic acid by treatment of the ketone with an appropriate amine under reducing conditions:



Examples of Y-region precursors which contain a carbon
 30 chain interrupted by heteroatoms include those derived from poly(alkylene oxides), such as poly(ethylene oxide),

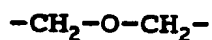
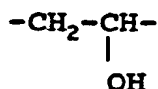
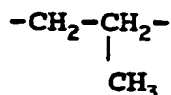
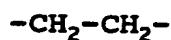
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poly(propylene oxide), and poly(butylene oxide) and poly(alkylene imines), such as poly(ethylene imine), poly(propylene imine) and poly(butylene imine). Among these, poly(ethylene oxide) and poly(ethylene imine) are preferred
 5 due to hydrophilicity considerations.

Specific examples of Y-region precursors which contain carbon chains interrupted by heteroatoms include:

- $\text{HO}_2\text{CCH}_2\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CO}_2\text{H}$, where $n = 0$ to 8;
 $\text{HO}_2\text{CCH}_2\text{CH}_2\text{O}(\text{CH}_2\text{CH}_2\text{CH}_2\text{O})_n\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$, where $n = 0$ to 4;
 10 $\text{HO}_2\text{CCH}_2\text{N}(\text{CH}_2\text{CH}_2\text{N})_n\text{CH}_2\text{CO}_2\text{H}$, where $n = 0$ to 8; and
 $\text{HO}_2\text{CCH}_2\text{CH}_2\text{N}(\text{CH}_2\text{CH}_2\text{CH}_2\text{N})_n\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$, where $n = 0$ to 4.

The following are some additional specific Y groups which are contemplated:



- 15 Each of the molecules of the present invention can exist as enantiomers due to the chiral carbon to which the α -amino group is attached. Both D and L isomers, as well as mixtures of the two (e.g., a racemic mixture) are contemplated as part of this invention. The optically active molecules may be
 20 prepared by resolving mixtures thereof or may be prepared from

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optically active precursors. The L isomers are preferred. When the substituted Y groups are capable of optical activity, additional isomers will be possible, and these are also part of the present invention, both individually and as a mixture.

5 The functional group of the Z segment serves to bind a second biomolecule or bioaffecting molecule to the crosslinker. The reactive group of the Z region is preferably: 2-dithiopyridyl, 4-dithiopyridyl, thiolacetate, or maleimide. Each of these Z groups is preferably
10 unsubstituted, but could be substituted by 1 to 3, preferably 1, substituent selected from: halogen (e.g. Cl, Br, I, F), nitro, hydroxy, and C₁₋₄ alkyl.

 In the case of dithiopyridyl, a stable disulfide bond will be formed between the present crosslinkers and a thiol
15 appended from the molecule to which it is linked. The thiolacetate functionality permits the introduction of a masked thiol into the carrier-linker adduct. Liberation of the acetate group, e.g., via reduction or base-catalyzed hydrolysis provides a free mercaptan which can then form a
20 stable linkage with a molecule which contains a thiol reactive group, such as, e.g., dithiopyridyl or maleimide functionalities. The maleimide group permits the formation of a stable sulfide upon reaction with a molecule bearing a mercaptan.

25 The compounds of the present invention can exist as salts. These salts also form part of the present invention. The salt of the α -amino group may be any conventional salt known to an organic chemist.

 Preferred salts are: hydrochloride, hydrobromide,
30 p-TsOH, hydroiodide, etc.

 In a preferred embodiment Y is -CH₂- because then the present crosslinkers may be directly prepared by linking the X

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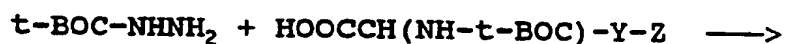
and Z units to cysteine. $\text{NH}_2\text{NH}-$ is preferred for X, and dithiopyridyl is preferred for Z. The compound, wherein Y is $-\text{CH}_2-$, X is $\text{NH}_2\text{NH}-$ and Z is 2-dithiopyridyl, is S-(2-thiopyridyl)-cysteine hydrazide, referred to herein as TPCH, and is a particularly preferred embodiment. It can exist as D and L isomers, and the L is preferred. It can also exist as a salt, and the trihydrochloride is preferred. The synthesis of this compound is discussed hereinbelow.

Synthesis

The present crosslinkers may be synthesized via coupling of an amine-containing compound, such as H_2NNH_2 , $\text{H}_2\text{NNHCONHNH}_2$, $\text{H}_2\text{NNHCONH}_2$, $\text{H}_2\text{NO}(\text{CH}_2)_n\text{NH}_2$, $\text{H}_2\text{NOCO}(\text{CH}_2)_n\text{NH}_2$, or $\text{H}_2\text{N}(\text{CH}_2)_n\text{NH}_2$ with a carboxylic acid. The carboxylic acid may be derived from an amino acid precursor. The amino acid precursor may be N-protected, and the amine-containing compound may be protected at one of the $-\text{NH}_2$ groups. Suitable N-protecting groups include, e.g., t-butyloxycarbonyl (t-BOC), carbobenzoxy (CBZ), 9-fluorenylmethoxycarbonyl (Fmoc), o-nitrophenylsulfenyl, or p-nitro-2-pyridinesulfenyl. t-BOC is preferred.

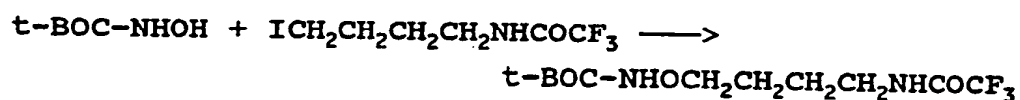
The carboxyl group of the amino acid precursor may be derivatized to form a more reactive group, such as, e.g., an acyl halide, in particular an acyl chloride.

The coupling of the amino acid precursor and the amine-containing compound may be mediated by a dehydrating agent, such as, a carbodiimide, in particular N,N'-dicyclohexylcarbodiimide (DCC). An example of one coupling reaction is shown below:



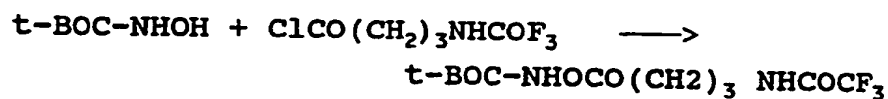
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In the case where X is $\text{H}_2\text{NO}(\text{CH}_2)_n\text{NH}-$, the synthesis of the X region precursor may be accomplished by the alkylation of a N-hydroxycarbamate, such as, e.g., t-butyl-N-hydroxycarbamate, with a reagent having the structure $\text{L}(\text{CH}_2)_n\text{NH}_2$, where L is a leaving group and the $-\text{NH}_2$ group may be protected. Suitable leaving groups include, e.g., the halides, and iodide is preferred. Suitable $-\text{NH}_2$ protecting groups are those given above, and again, t-BOC is preferred. An example of one preparation of one X-region precursor from a reagent prepared from γ -aminobutyric acid is shown below:



In this case, the trifluoroacetamide protection can be selectively cleaved by conventional methods and the resulting free amino group coupled with an amino acid optionally containing the Y and Z linker segments, as described above.

When X is $\text{H}_2\text{NOCO}(\text{CH}_2)_n\text{NH}-$, the precursor for the X region may be prepared by the acylation of a N-hydroxycarbamate, such as, e.g., t-butyl-N-hydroxycarbamate, with a reagent having the structure $\text{L}'\text{CO}(\text{CH}_2)_n\text{NH}_2$, where L' is a group such as $-\text{OH}$ or a halide and the $-\text{NH}_2$ group may be protected as described above. When L' is $-\text{OH}$ the reaction may be mediated with a dehydrating agent as described above. An example of one preparation of one X-region precursor from a reagent prepared from γ -aminobutyric acid is shown below:



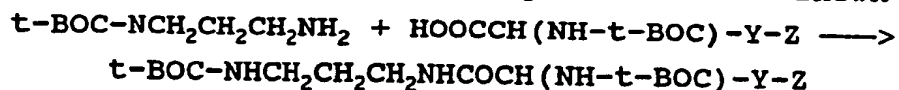
Again, the trifluoroacetamide protection can be removed and the free amino group coupled with an amino acid.

When X is $\text{H}_2\text{N}(\text{CH}_2)_n\text{NH}-$, the crosslinkers can be prepared by the reaction of the amino acid, which may or may not contain

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linker sections Y and Z, with a diamine. The diamine may be partially protected with any of the above-mentioned protecting groups, preferably t-BOC, and the coupling may be mediated by any of the above-mentioned dehydrating agents, preferably DCC.

5 One example of one coupling reaction is shown below:



In all of the above cases, the amine protecting groups can be removed as a final step. For example, the t-BOC protection
10 can be removed as a final step by treatment with anhydrous HCl or trifluoroacetic acid.

The coupling of the segments of the linker follows conventional methodology and may vary with the incorporation of some of the options outlined above. A preferred central building
15 block for the linker is cysteine, which provides not only the crucial α -amine but a reactive sulfhydryl group. For example, the above-mentioned diacid precursors for the Y region can be reduced and halogenated selectively at one end, and the resulting active halo compound can be used to alkylate the thiol of
20 cysteine, which is optionally protected at the amine and carboxyl groups. The second carboxylic acid function of a diacid Y-region precursor may also be protected. Suitable amine protecting groups are those mentioned above, with t-BOC being preferred. Suitable carboxy protecting groups include, e.g., benzyl ester,
25 t-butyl ester, ethyl ester, and methyl ester.

Deprotection of the cysteine-derived carboxy and coupling with any of the groups enumerated under the discussion of X given above may be effected as discussed above.

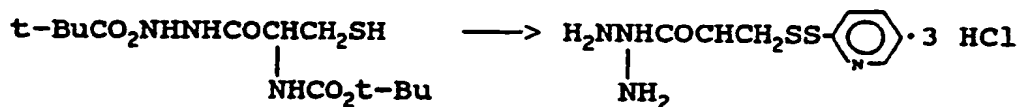
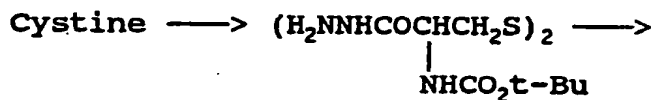
The X-amino acid-Y conjugate may then be coupled with
30 reagents containing the Z functionalities discussed above. The free carboxy functionality of Y may be transformed into a more nucleophilic and hydrophilic residue, such as an acyl hydrazide,

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e.g., via carb diimide-mediated coupling with carbohydrazide. Subsequent reaction with any of several conventional reagents, e.g., SPDP, will introduce the Z portion of the linker. A final deprotection step may be necessary.

5 Preferably, the X, Y and Z groups are selected so that they are not readily chemically reactive among each other. Dithiopyridyl is unreactive toward and thus compatible with all X regions. Thiolacetate is compatible with $\text{H}_2\text{NO}(\text{CH}_2)_n\text{NH}-$ and $\text{H}_2\text{NOCO}(\text{CH}_2)_n\text{NH}-$ at pH up to 4 and with $\text{H}_2\text{N}(\text{CH}_2)_n\text{NH}-$ at a pH from
 10 about 4 to about 7. Maleimide is compatible with all of the X regions in the pH ranges at which the X regions are substantially protonated.

S-(2-thiopyridyl)-L-cysteine hydrazide (TPCH) is the particularly preferred crosslinker. One synthesis of TPCH is
 15 outlined below.



L-Cystine alkyl ester dihydrochloride can be prepared from commercially available L-cystine (Aldrich Chemical Co.)
 20 by treatment with thionyl chloride in an alcohol. The amino ester may be protected with di-tert-butyl dicarbonate, affording the bis(tert-butyl urethane) derivative. The corresponding bis(hydrazide) may be prepared by treatment with excess hydrazine and may be protected at the hydrazide
 25 functionality by reaction with di-tert-butyl dicarbonate. The disulfide linkage can be reductively cleaved with, e.g., zinc dust in aqueous acetic acid, providing the free thiol. The dithiopyridyl functionality may be introduced by reaction with 2,2'-dipyridyl disulfide. Finally the removal of the two

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urethane groups provides TPCH as a hygroscopic white crystalline powder.

Uses of the Crosslinkers

The heterobifunctional crosslinkers of the present invention may be used to couple a variety of compounds.

It is preferred that the present crosslinkers are coupled via the X region to biomolecules and bioaffecting molecules which contain an amine reactive group or a group which can be converted to an amine reactive group, such as a carbohydrate group. By "biomolecules" and "bioaffecting molecules" is meant glycoproteins, glycolipids, nucleic acids, monosaccharides, and polysaccharides and drugs. Glycoproteins include antibodies, lectins, enzymes, and response modifiers.

For example, the present X groups can react with a number of different functional groups including aldehydes, hemiacetals, ketones, and carboxy groups. When the present crosslinkers are coupled to carboxy groups via X, the coupling reaction may be mediated by a dehydrating agent, such as a carbodiimide.

Examples of compounds which may be derivatized via aldehyde functions include glycoproteins (see, e.g., Debray et al., J. Biol. Chem., vol. 250, 1955 (1975) and Bayer et al., Methods Biochem. Anal., vol. 26, 1 (1980)), such as antibodies (e.g., IgM; O'Shannessy, Int. Soc. Biorecognition Tech., vol. 3, pp. 4-6 (1988) and IgG; Redwell et al., Proc. Natl. Acad. Sci. USA, vol. 83, pp. 2632-2636 (1986)), lectins (e.g., ricin; Montfort, et al., J. Biol. Chem., vol. 262, pp. 5398-5403 (1987)), enzymes (e.g., alkaline phosphatase; Fosset, et al., Biochem., vol. 13, pp. 1783-1788 (1974)) and response modifiers (e.g., cobra venom factor; Vogel, et al., J. Immunol. Meth., vol. 73, pp. 203-220 (1984)); glycolipids, e.g., c r broside; nucleic acids, e.g., RNA; mono- or

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polysaccharides, e.g., gangliosides (Spiegel et al., Biochem. Biophys. Acta, vol. 687, 27 (1982)); and drugs, e.g., doxorubicin.

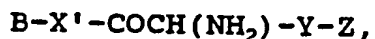
Examples of compounds which may be derivatized via a hemiacetal group include mono- or polysaccharides, such as oligosaccharides with a reducing terminus. The derivatization of doxorubicin via the C-13 keto group has been reported in Annals of the New York Academy of Sciences, volume 417, pp. 125-136 (1983). Compounds which may be derivatized via the carbodiimide-mediated reaction of a carboxy group include drugs such as succinylated T2-toxin.

The present crosslinkers may be coupled to molecules via the X-region by any of the conventional methods, such as those described in O'Shannessy, Int. Soc. Biorecognition Tech. Commun., vol. 3, pp. 4-6 (1988), which is incorporated herein by reference. For example, the present crosslinkers may be coupled to the oxidized carbohydrate region of an antibody. The carbohydrate region of antibody may be oxidized by any conventional oxidant such as periodate, periodic acid, para-periodic acid, or metaperiodate. In addition, enzymatic oxidation with, e.g., galactosidase as described in Biochem. Biophys. Acta, vol. 800, pp. 291300 (1984), is also suitable.

The present crosslinkers may be coupled to the oxidized carbohydrate region of an antibody by first oxidizing the carbohydrate region and then reacting the oxidized antibody with the crosslinker. Alternatively, the coupling may be accomplished by oxidizing the antibody in the presence of the present crosslinker. The latter method results in fewer undesirable side reactions which can decrease the binding function of the antibody and, thus, is preferred.

The product of the coupling of the present crosslinkers with either a carbonyl or hemiacetal group of a biomolecule may be represented by the formula:

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in which X' is =NNH-, =NNHCONHNNH-, =NNHCONH-, =NO(CH₂)_nNH-, =NOCO(CH₂)_nNH-, and =N(CH₂)_nNH-, where n is an integer of 2 to 6, Y and Z are defined as above, and B is a biomolecule or
5 bioaffecting molecule.

Thus, the reaction of the X-region of the present crosslinkers with a carbonyl or hemiacetal functional groups results in the formation of a carbon-nitrogen double bond. When X is H₂N(CH₂)_nNH-, the linkage is an imine. It may be
10 necessary to stabilize the imine bond, and this may be accomplished by any conventional method, such as reduction. Suitable reducing agents include NaBH₄ and Na(CN)BH₃.

The present crosslinkers may be coupled to molecules via the Z region by a number of different methods. For example,
15 when Z is 2- or 4-dithiopyridyl, the crosslinker may be coupled directly to molecules which contain a free mercapto group via a disulfide exchange reaction. The progress of the coupling may be monitored by detecting the liberated pyridine-thione.

Alternatively, the crosslinker where Z is a dithiopyridyl may be reacted with a molecule which possesses a disulfide group in the presence of a reducing agent, such as NABH₄ or dithiothreitol (DTT). The disulfide group of the molecule to be coupled with the crosslinker may be created by first
25 linking the molecule to be coupled to another molecule which contains a disulfide group. For example, a molecule may be first coupled to TPCH or SPDP via the X region or succinimidyl group, respectively, to obtain a molecule with a dithiopyridyl group, and then reacted with another molecule already
30 derivatized with one of the present crosslinkers where Z is dithiopyridyl in the presence of a reducing agent.

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In another embodiment, either a free thiol group originating from Z = thiolacetate or from the reduction of Z = thiopyridyl may be reacted with a molecule which contains either a disulfide group, maleimide group, or halide, such as iodide or bromide. Again, the disulfide group on the molecule to be coupled to the present crosslinker may be created by first reacting the molecule to be coupled with a disulfide-containing molecule, such as TPCH or SPDP. In addition, when the Z-region of the crosslinker is maleimide, the crosslinker may be linked to molecules which contain a free sulfhydryl group by direct reaction to form a sulfide or with molecules which contain a disulfide group by reaction in the presence of a reducing agent.

The present crosslinkers exhibit numerous advantages over those of the prior art. In particular, the NH_2 -containing reactive groups of X permit the site-specific labelling of biomolecules and bioaffecting molecules. In particular, the present crosslinkers can be used to form site-specific labelled antibodies which retain a high degree of binding.

For example, TPCH can be linked to human IgM antibody 1688 which has had its carbohydrate region oxidized by either mild periodate or enzymatic oxidation. Derivatization of the antibody with as many as 35 TPCH crosslinker molecules did not affect antibody binding to the tumor antigen. In contrast, the introduction of only 16 SPDP molecules per IgM antibody resulted in virtually a complete loss of the antibody binding function.

Further, when approximately four cobra venom factor molecules were coupled to antibody 1688 via TPCH, the antigen binding function decreased by less than a factor of 1.5. On the other hand, coupling about the same number of cobra venom molecules via SPDP to antibody 1688 resulted in a decrease in antibody binding function by a factor of 40. These data demonstrate that the novel site-specific heterobifunctional

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crosslinking reagents of the present invention permit the synthesis of immunoconjugates with unimpaired antigen binding capabilities.

The present crosslinkers are also efficiently and rapidly incorporated into biomolecules such as antibodies. For example, Figure 1 compares the degree of incorporation of TPCP and the structurally most similar crosslinker from Japanese Patent Application J63-57569, S-(2-thiopyridyl)-mercaptopropionic acid hydrazide (TPMPH), into human monoclonal IgM antibody 1688 under two different coupling conditions. In both cases, TPCP is incorporated into the antibody in a significantly greater amount.

Figure 2 shows the rates of incorporation of TPCP and TPMPH into human monoclonal IgM antibody 1688 under identical coupling conditions. The results presented in Figure 2 demonstrate that the incorporation of comparable amounts of crosslinker molecules per antibody requires approximately a 3-fold increase in time for TPMPH as compared for TPCP. Consequently, the use of TPCP permits a reduction in the exposure of the antibody to the oxidizing agent, in this case sodium periodate. As noted above extended exposure of the antibody to the oxidizing agent produces undesired side reactions of the primary amino groups of the antibody with the generated aldehyde residues and decreases the antigen binding capability. Since the use of TPCP reduces the time that the antibody is exposed to the oxidizing agent, it reduces the amount of undesired side reactions and ensures high antibody binding activity.

In addition, the water solubility of TPCP is significantly higher than that of TPMPH. In distilled water TPCP can be easily dissolved to a concentration of 1M (5mg of TPCP (14.2 μ moles)/14.2 μ l), whereas the maximum solubility of TPMPH is 150 mM (5mg of TPMPH (2.1 μ moles)/145 μ l).

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In another embodiment, the present invention relates to biomolecules which have already been coupled to the present crosslinkers. Any of the previously mentioned biomolecules may be derivatized with the present functional groups.

- 5 Antibodies are the preferred molecules to be linked by the present crosslinkers, and human monoclonal IgM antibody 1688 is particularly preferred.

Since the sulfhydryl groups of the Z portion of the present crosslinkers are protected, the present crosslinkers
10 are stable and can be stored and thereby are useful reagents for kits. The present crosslinkers may be contained in a kit in either a completely unreacted form or already attached to a molecule via the X group. The exact contents of a particular kit will vary depending on the intended use and whether the
15 crosslinker is attached to a molecule, and if so, the nature of the attached molecule.

All publications cited herein are incorporated herein by reference.

Other features of the invention will become apparent in
20 the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Preparation of N,N'-di-(tert-Butyloxycarbonyl)-L- 25 Cystine Dihydrazide:

A solution of 2.47 g (5.28 mmol) of N,N'-di(tert-butylloxycarbonyl)-L-cystine dimethyl ester in 50 ml methanol was treated dropwise with 10 ml anhydrous hydrazine at room temperature. Th solution was maintained at room temperature
30 f r two hours over which time a fine white material precipitated. Th solution was cooled to 0°C for 30 minutes and the pr duct was collected by filtration and washed with

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ice-cold methanol to provide 2.14 g (86.8%) of white crystals. ^1H NMR (CD_3COCD_3): 9.0 (br s, 1H, exchangeable with D_2O), 5.62 (br d, 1H, exchangeable with D_2O), 4.84 (m, 1H), 3.5 (br, 1H, exchangeable with D_2O), 2.92 (br, 2H), 1.45 (s, 9H).

5 Preparation of Tetra-(tert-Butyloxycarbonyl-L-Cystine Dihydrazide:

A suspension of 10.40 g (22.22 mmol) of N,N'-di-(tert-butyloxycarbonyl)-L-cystine dihydrazide in 180 ml ethanol was treated with 20 ml diisopropylethylamine and
10 warmed to reflux. The suspension dissolved upon warming, and 9.70 g (44.44 mmol) of di-(tert-butyl)dicarbonate was added portionwise. The clear, colorless solution was refluxed for thirty minutes and then allowed to cool to room temperature. After 20 minutes the product began to crystallize from
15 solution. The mixture was stored at room temperature for one hour, then cooled to 0°C for one hour. The white crystalline product was collected by filtration and was washed with ice-cold ethanol. 10.80 g (72.8%) were obtained. $^1\text{HNMR}$ was very complex due to the apparent restricted rotation about the
20 three amide-type bonds. At least three rotamers can be identified in the spectrum.

Preparation tert-Butyloxycarbonyl-L-Cystine Hydrazide:

Zinc dust (3g) was added in portions over two hours to a suspension of 10.80 g (16.17 mmol) tetra(tert-
25 -butyloxycarbonyl)-L-cystine dihydrazide in 40 ml acetic acid containing 6 ml water. Gradually the suspension dissolved and after two hours the solution was concentrated under reduced pressure, and the residue was partitioned between methylene chloride and saturated aqueous sodium bicarbonate. The
30 methylene chloride was dried over sodium sulfate and concentrated to a viscous glass. The yield was 10.0 g (92.6%). ^1H NMR was very complex due to the apparent restricted

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rotation about the three amide-type bonds. At least three rotamers can be identified in the spectrum.

Preparation of Di-(tert-Butyloxycarbonyl)-S-(2-Thiopyridyl)-L-Cysteine Hydrazide:

5 6.57 g (29.85 mmol) of 2,2'-dipyridyl disulfide was added portionwise to a solution of 5.00 g (14.93 mmol) di-(tert-butyloxycarbonyl)-L-cysteine hydrazide in 75 ml methanol at room temperature. This solution was maintained at room temperature for 24 hour, then concentrated in vacuo to a
10 yellow syrup. The crude product was taken up in 400 ml methanol and 20g silica gel (32-60 μ m) was added. The crude product was absorbed onto the silica gel by evaporation of the solvent, and the impregnated gel was placed atop a 95 mm i.d. x 55mm column of silica gel (32-60 μ m). The product was
15 isolated by eluting with 35 ethyl acetate: 65 hexanes. Fractions (100 ml each) containing product were pooled and concentrated to provide 3.5 g (52.8%) of a colorless glass. ^1H NMR (CDCl_3) was complex due to the presence of at least two rotamers in solution: 9.54 (br, 0.25H, exchangeable with D_2O),
20 8.60 (br, 0.75H, exchangeable with D_2O), 6.553 (br, 1H, exchangeable with D_2O), 5.786 (m, 0.5H), 4.925 (m, 0.5H), 4.526 (br s, 1H), 3.384 (m, 1H), 2.910 (m, 1H), 1.456 (br s, 9H), 1.408 (br, 9H).

Preparation of S-(2-Thiopyridyl)-L-Cysteine Hydrazide
25 Trihydrochloride:

(TPCH) A solution of 1.15 g (2.58 mmol) of di-(tert-butyloxycarbonyl)-S-(2-thiopyridyl)-L-cysteine hydrazide in 15 ml ethyl acetate was cooled to 0°C and 25 ml of a saturated solution of anhydrous hydrogen chloride in ethyl
30 acetate was added slowly. After 30 minutes a white crystalline material began to separate. The mixture was stirred at room temperature for 4 hours, then filtered under argon, and washed with ethyl acetate, and dried under argon,

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th n under vacuum to provide 830 mg (91%) of hygroscopic white crystals. ^1H NMR (D_2O): 8.63 (m, 1H), 8.31 (m, 1H), 8.14 (m, 1H), 7.74 (m, 1H), 4.47 (m, 1H), 3.45 (m, 2H) ppm. ^{13}C NMR ($\text{DMSO}-d_6$): 166, 157, 149, 140, 122, 121, 50, 21 ppm.

5 Derivatization of Antibody with TPCH:

Human IgM (2.5 mg) in 0.1 M sodium acetate, pH 5.5 (1 mg IgM/ml) was oxidized in the presence of 10.7 mg (50 μmoles) NaIO_4 and 7 mg (19.8 μmoles) of TPCH. After 20 minutes at 0°C the reaction mixture was chromatographed on a Sephadex G-25
10 column to remove access NaIO_4 and uncoupled TPCH. The derivatized IgM molecules were eluted with 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.5, and stored at 4°C . The extent of modification was determined to be 17 TPCH molecules per IgM by monitoring the release of
15 pyridine-2-thione at 343 nm during incubation of the derivatized antibody in the presence of 10 mM dithiothreitol as described in J. Carlsson, H. Drevin, and R. Axen, Biochem. J., vol. 173, pp. 723-737 (1978), which is incorporated herein by reference.

20 Conjugation of SPDP-Derivatized Cobra Venom Factor With TPCH-Derivatized IgM Antibody:

Cobra venom factor (CVF) was derivatized with 2 molecules of N-succinimidyl-3-(2pyridyldithio)propionate (SPDP) per CVF molecule as described in E.C. Petrella, S.D. Wilkie, C.A.
25 Smith, A.C. Morgan, Jr., and C.-W. Vogel, J. Immunol. Meth., vol. 104, pp. 159-172 (1987), which is incorporated herein by reference. After removal of excess SPDP by size exclusion chromatography the modified CVF was incubated in 0.1 M sodium acetate, 0.1 M sodium chloride, pH 4.5, containing 50 mM
30 dithiothreitol to generate free sulfhydryl residues. After 20 minutes at 25°C , dithiothreitol was removed by size exclusion chromatography and 1.85 mg of free sulfhydrylcontaining CVF was incubated with 1 mg of TPCH-derivatized IgM at a final

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protein concentration of 1.25 mg/ml in 0.1 M sodium phosphate ,
0.1 M sodium chloride, pH 7.5, at 25°C. After 24 hours free
cobra venom factor was removed by size exclusion
chromatography on a TSK-4000 HPLC column. The immunoconjugate
5 fraction contained IgM and CVF covalently coupled at a
stoichiometry of 1:3.2 as determined by densitometry of the
gel electrophoretically separated immunoconjugate fraction.

Derivatization of Antibody with SPDP:

Human IgM (2.5 mg) at a concentration of 1 mg/ml in 0.1 M
10 sodium phosphate, 0.1 M sodium chloride, pH 7.5, was incubated
with 8.8 µg of SPDP for 30 minutes at 25°C. The reaction
mixture was then chromatographed on a Sephadex G-25 column to
remove excess SPDP. The SPDP-modified human IgM was eluted
with 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.5,
15 and stored at 4°C. The extent of modification was determined
to be 8 SPDP molecules per antibody using the method described
above for TPCH derivatization. When human IgM was incubated
with 36.7 µg/ml of SPDP for 30 minutes at 25°C, a ratio of 16
SPDP molecules per antibody was obtained.

20 Conjugation of SPDP-Derivatized Cobra Venom Factor to
SPDP-Derivatized IgM Antibody:

The conjugation was carried out under identical
conditions as described above for TPCH-derivatized antibody.
Using human IgM derivatized with 8 SPDP molecules, the
25 resulting immunoconjugates contained IgM and CVP at a ratio of
1:5.6.

Comparison of the Binding Function of TPCH- and SPDP-
Derivatized IgM Antibody:

The binding functions of TPCH- and SPDPderivatized IgM
30 antibodies were determined by a radioimmunoassay. The
principle of the assay consists of measuring the ability of an

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unmodified or modified IgM 1688 antibody to compete for antigen with a radiolabelled (otherwise unmodified) IgM 1688 antibody. The radiolabelled antibody is present in a constant amount, whereas the unlabelled (unmodified or modified) antibody is added in varying amounts. The inhibition of the binding of the radiolabelled antibody is measured.

The antigen is obtained from a 20% NH_4SO_4 precipitate of colon carcinoma cells, WiDr, and coated at a concentration of 8 $\mu\text{g/ml}$ for 15 hours at 4°C onto polystyrene microtiter plates. The antigen coated wells are blocked with 250 μl of 1% (weight/volume) fish gelatin and phosphate buffer saline (PBS) (pH 7.2) for 1 hour at room temperature, and then washed twice with 0.05% (volume/volume) Tween 20®, and 5% (volume/volume) glycerol. Thereafter 50 μl of unlabelled and 50 μl of radiolabelled IgM 1688 antibody in PBS (pH 7.2) containing 1% (weight/volume) bovine serum albumin (BSA) (Buffer A) is added, mixed, and incubated at 4°C. After 15 hours, the wells are washed three times with Buffer A and then counted for radioactivity.

20 a) Retention of antigen binding capability after derivatization with 17 TPCN molecules:

The amount of 1688 IgM required to achieve 50% inhibition in the radioimmunoassay was 4.0 $\mu\text{g/ml}$ for unmodified IgM and 5.5 $\mu\text{g/ml}$ for TPCN-derivatized IgM.

25 b) Retention of antigen binding capability after derivatization with 16 and 8 SPDP molecules:

The amount of 1688 IgM required to achieve 50% inhibition in the radioimmunoassay was 4.0 $\mu\text{g/ml}$ for unmodified IgM and 13.3 $\mu\text{g/ml}$ for IgM derivatized with 8 SPDP molecules. When derivatized with 16 SPDP molecules 50% inhibition could not be achieved even at a concentration of 40 $\mu\text{g/ml}$ of derivatized IgM (12% inhibition).

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c) Retention of antigen binding capability after coupling of 3.2 molecules of CVF to 1688 IgM derivatized with 17 TPCH molecules:

The amount of IgM in the IGM-CVF conjugates required to achieve 50% inhibition was 6.0 $\mu\text{g/ml}$.

d) Retention of antigen binding capability after coupling of 5.6 molecules CVF to 1688 IgM derivatized with 8 SPDP molecules:

Even at a concentration of 40 $\mu\text{g/ml}$ IgM in the IgM-CVF conjugates not more than 12% inhibition could be achieved.

Efficiency of Incorporation of TPCH and TPMPH into Human IgM in the Presence of 20 mM Sodium Periodate:

Human IgM was oxidized in 1.25 ml of 0.1 M sodium acetate, pH 5.5, in the presence of 5.3 mg sodium periodate with either 6.9 mg (20 μmoles) of TPCH trihydrochloride (M.W. = 353.5 daltons) or 4.4 mg (20 μmoles) of TPMPH (M.W. 229.3 daltons) at 0°C. Aliquots (250 μl) of the reaction mixture were applied to Sephadex G-25 column chromatography to remove excess sodium periodate and uncoupled TPCH or TPMPH. The derivatized IgM molecules were eluted with 0.1 M sodium phosphate, 0.1 M sodium chloride, pH 7.5. The extent of modification was determined as described above.

Efficiency of Incorporation of TPCH and TPMPH into Human IgM in the Presence of 1mM Sodium Periodate:

Human IgM (2.5 mg) was oxidized in the presence of 0.27 mg sodium periodate under identical conditions as described above. The extent of derivatization was determined after 20 minutes of incubation using the procedure described above.

Obviously, numerous modifications and variations of the present invention are possible in light of the above

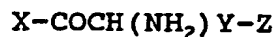
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teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

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Claims:

1. A molecule having the formula:



wherein X is one member selected from the group consisting of
 5 $\text{H}_2\text{NNH}-$, $\text{H}_2\text{NNHCONHNH}-$, $\text{H}_2\text{NNHCONH}-$, $\text{H}_2\text{NO}(\text{CH}_2)_n\text{NH}-$, $\text{H}_2\text{NOCO}(\text{CH}_2)_n\text{NH}-$,
 and $\text{H}_2\text{N}(\text{CH}_2)_n\text{NH}-$, where n is an integer of 2 to 6;

Y is a divalent group having 1 to 20 carbon atoms, which
 may be interrupted by heteroatoms, and may be substituted with
 hydroxyl, carboxy, sulfonate, phosphonate, or quaternary
 10 ammonium; and

Z is one member selected from the group consisting of
 dithiopyridyl, thiolacetate, and maleimide.

2. The molecule of Claim 1, wherein X is $\text{H}_2\text{NNH}-$.

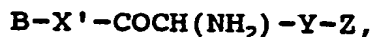
3. The molecule of Claim 1, wherein Y is $-\text{CH}_2-$.

- 15 4. The molecule of Claim 1, wherein Z is 2-dithiopyridyl
 or 4-dithiopyridyl.

5. The molecule of Claim 4, wherein Z is 2-dithiopyridyl.

6. The molecule of Claim 1, which is S-(2-thiopyridyl)-
 L-cysteine hydrazide.

- 20 7. A molecule having the formula:



wherein X' is one member selected from the group
 consisting of $=\text{NNH}-$, $=\text{NNHCONHNH}-$, $=\text{NNHCONH}-$, $=\text{NO}(\text{CH}_2)_n\text{NH}-$,
 $=\text{NOCO}(\text{CH}_2)_n\text{NH}-$, and $=\text{N}(\text{CH}_2)_n\text{NH}-$, where n is an integer of 2 to
 25 6;

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Y is a divalent-group having 1 to 20 carbon atoms, which may be interrupted by heteroatoms, and may be substituted with hydroxyl, carboxy, sulfonate, phosphonate, or quaternary ammonium;

5 Z is one member selected from the group consisting of dithiopyridyl, thiolacetate, and maleimide; and

B is selected from the group consisting of glycoproteins, glycolipids, nucleic acids, monosaccharides, and polysaccharides.

10 8. The molecule of Claim 7, wherein said biomolecule is an antibody.

9. The molecule of Claim 8, wherein said antibody is human monoclonal IgM antibody 1688.

10. The molecule of Claim 7, wherein XI is =NNH-.

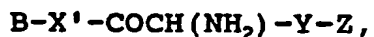
15 11. The molecule of Claim 7, wherein Y is -CH₂-.

12. The molecule of Claim 7, wherein Z is 2-dithiopyridyl or 4-dithiopyridyl.

13. The molecule of Claim 7, wherein Z is 2-dithiopyridyl.

20 14. The molecule of Claim 7, wherein X' is =NNH-, Y is -CH₂-, and Z is 2-dithiopyridyl.

15. A kit, comprising a molecule having the formula:



wherein X' is one member selected from the group
25 consisting of =NNH-, =NNHCONHNH-, =NNHCONH-,
=NO(CH₂)_nNH-, =NOCO(CH₂)_nNH-, and =N(CH₂)_nNH-, where n is an integer of 2 to 6;

Y is a divalent group having 1 to 20 carbon atoms, which may be interrupted by heteroatoms, and may be substituted with

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hydroxyl, carboxy, sulfonate, phosphonate, or quaternary ammonium;

Z is one member selected from the group consisting of dithiopyridyl, thiolacetate, and maleimide; and

5 B is selected from the group consisting of glycoproteins, glycolipids, nucleic acids, monosaccharides, and polysaccharides.

16. The kit of Claim 15, wherein said biomolecule is an antibody.

17. The kit of Claim 15, wherein said antibody is human
10 monoclonal IgM antibody 1688.

18. The kit of Claim 15, wherein X' is =NNH-.

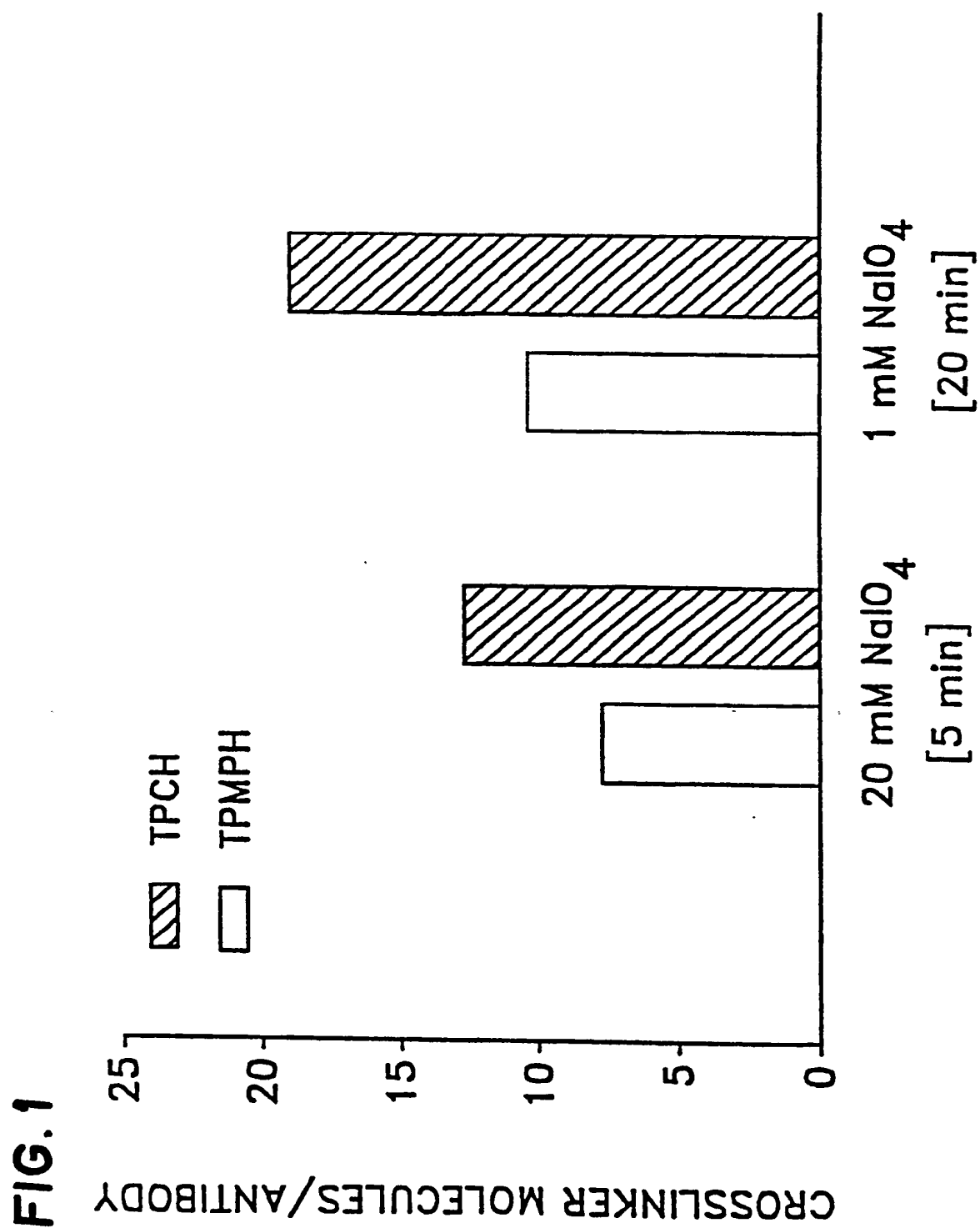
19. The kit of Claim 15, wherein Y is -CH₂-.

20. The kit of Claim 15, wherein Z is 2-dithiopyridyl or 4-dithiopyridyl.

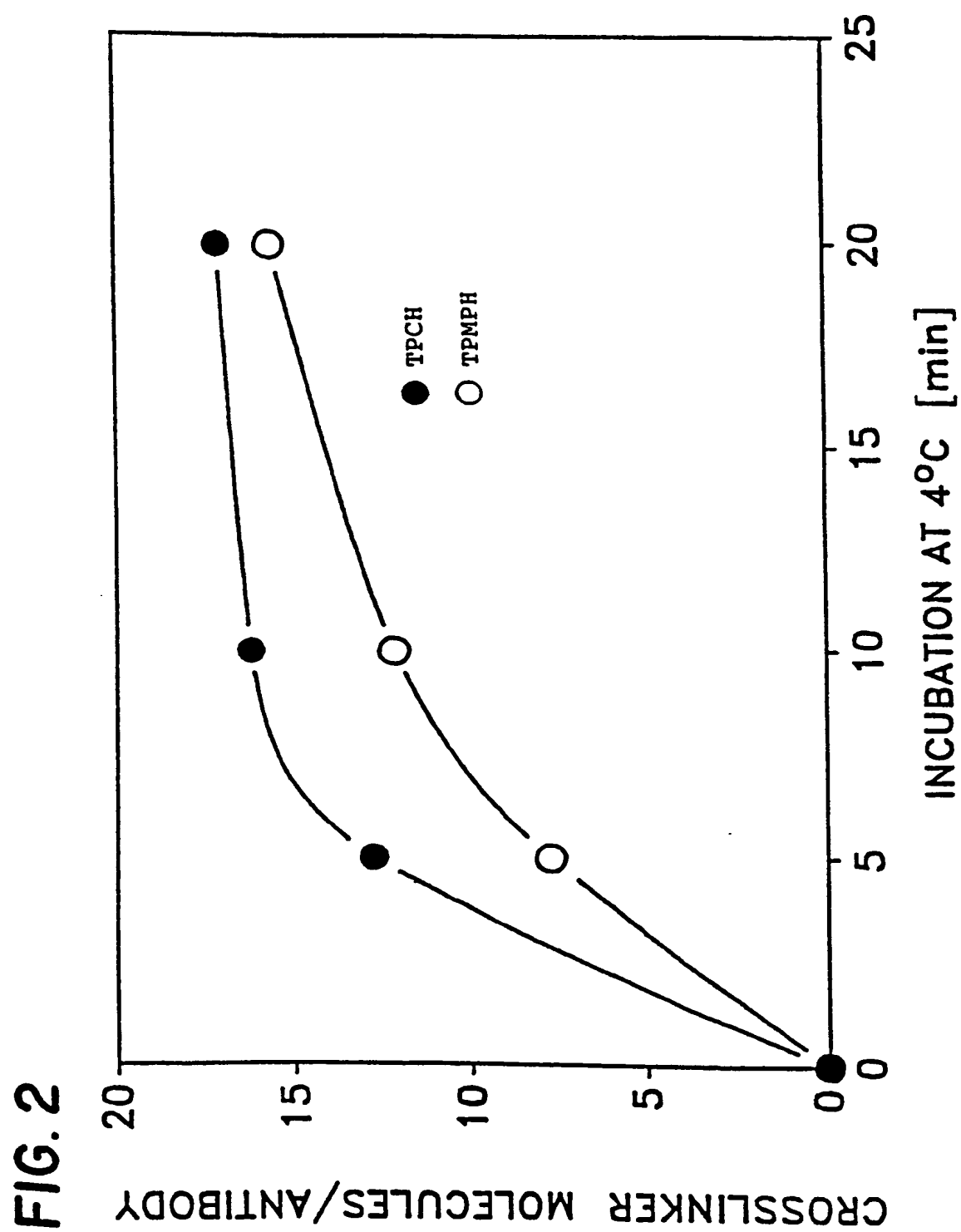
15 21. The kit of Claim 15, wherein Z is 2-thiopyridyl.

22. The kit of Claim 15, wherein X' is =NNH-, Y is -CH₂-, and Z is 2-dithiopyridyl.

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INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/01201**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(5): C07D 207/46, C07D 207/48, C07D 213/71, A61K 39/00, A61K 37/00
U.S. CL.: 546/291, 424/85.91, 436/547, 436/548

G01N 33/53

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System

Classification Symbols

U.S. CL.

546/291, 424/85.91, 436/547, 436/548

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Chemical Abstracts On-Line Search

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category ⁸	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Chemical Abstracts, Volume 95, No.7, issued 1981, August 17, (Columbus, Ohio, U.S.A.), Pele C.S. Chong et al., "A New Heterobifunctional Cross-linking Reagent For The Study Of Biological Interactions Between Proteins. I. Design, Synthesis, And Characterization", See page 310, column 2, The Abstract No. 57582g, J. Biol. Chem. 1981, 256 (10), 5064-70 (Eng).	1-22
Y,P	The Sigma Chemical Company Catalog, 1990, (Sigma Chemical Company, P.O. BOX 14508, St. Louis, Missouri, U.S.A. 63178-9916), page 234, Cat. No. B5148, N,N ¹ -Bis(--BOC)-L-CYSTINE.	1-22
Y	Derwent Abstracts, Volume 88, issued 1988, March 13, (Tokyo, Japan), Teijin KK, "Bridging agents - comprises hydrazine compounds with active disulphide group and hydrazide group in the same molecule to introduce active group in glycosaccharide, the abstract no. 88-108817/16, J63057568-A, (Japanese).	1-22

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATE

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

18 APRIL 1990

25 MAY 1990

International Searching Authority

ISA/US

Signature of Authorized Officer

GARY L. KUNZ
GARY L. KUNZ

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4,797,491, (NITECKI), 10 January 1989, (see abstract).	1-22
A P	US, A, 4,880,935, (THORPE), 14 November 1989, (see columns 1-3).	1-22
A	US, A, 4,671,958 (RODWELL), 09 June 1987, (see columns 1-6).	1-22
A	Chemical Abstracts, Volume 94, No. 17, issued 1981, April 17, (Columbus, Ohio, U.S.A.), Keith E. Taylor ET AL., "A thiolotion", see page 377, column 2, The Abstract No. 135401t, <u>Biochem Int.</u> 1980, 1(4), 353-8 (Eng.).	1-22